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Formation of Elemental Sulfur by *Chlorella fusca* during Growth on L-Cysteine Ethylester[1]

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ABSTRACT

During growth on L-cysteine ethylester, *Chlorella fusca* (211-8b) accumulated a substance which contained bound sulfide, which could be liberated by reduction with dithioerythritol (DTE) as inorganic sulfide. This substance was extracted with hot methanol and purified by thin layer chromatography. This substance liberated free sulfide when incubated with mono- and dithiols, and thiocyanate was formed after heating with KCN. The isolated substance cochromatographed with authentic sulfur flower using different solvent systems for thin layer chromatography, high pressure liquid chromatography, and the identical spectrum with a relative λ_{max} at 263 nm was found. The chemical structure was confirmed by mass spectrometry showing a molecular weight of 256 m/e for the S_8 configuration. No labeled elemental sulfur was detected when the cells were grown on [^{35}S]sulfate and L-cysteine ethylester indicating the origin of elemental sulfur from L-cysteine ethylester. *C. fusca* seems to have enzymes for the metabolism of elemental sulfur, since it disappeared after prolonged growth into the stationary phase. Cysteine was formed from *O*-acetyl-L-serine and elemental sulfur in the presence of thiol groups and purified cysteine synthase from spinach or *Chlorella*.

Phototrophic organisms form elemental sulfur (S°) under certain growth conditions. This was observed for *Rhodospirillaceae*-like *Rhodopseudomonas* and *Rhodospirillum* (13), *Chromatiaceae*-like *Chromatium* (12), and *Chlorobiaceae*-like *Chlorobium* (12). In blue green algae such as *Oscillatoria* (23), *Aphanotheca*, (10) and other low algae (23), elemental sulfur was found too. Elemental sulfur was detected in eucaryots so far only in fungi (25) and the red algae *Erythrophyllum* (18) and *Ceramium* (17). The green alga *Chlorella fusca* metabolizes a variety of sulfur compounds using the sulfur atoms for growth (Krauss *et al.*, submitted). It was noticed during these studies that a precursor of sulfide (bound sulfide) accumulated during growth of *Chlorella* on certain derivatized cysteines, especially L-cysteine methylester and L-cysteine ethylester. This paper describes the characterization of this bound sulfide, which was identified as elemental sulfur.

MATERIALS AND METHODS

Organism and Growth Conditions. *Chlorella fusca* strain 211-8b of the algal collection of Göttingen (West Germany) was grown as described (Krauss *et al.*, submitted); however, the sulfate concentration was lowered to 2×10^{-4} M. If indicated, L-cysteine ethylester was added by sterile filtration to a final concentration of 3×10^{-4} M. Mass cultivation of algae was done in 10-L bottles at light intensities of 7000 lux. For growth on radio-labeled sulfate carrier-free sulfate was added to the growth medium to reach a specific activity of 1725 cpm/nmol sulfate. Growth on [^{35}S]sulfate was performed in 400-ml glass bottles connected to a Zn-acetate trap for labeled volatile sulfur compounds.

Isolation of Bound Sulfide. Bound sulfide was isolated from cultures growing in 10-L bottles on sulfate and L-cysteine ethylester when the culture reached an optical density of 0.6 at 680 nm. Cells were harvested by centrifugation and washed once with distilled H_2O. Bound sulfide was extracted with hot methanol. After incubation in methanol at 80°C for 10 min, the cells were centrifuged for 10 min at 5000*g* and the pellet was extracted again with hot methanol. The combined supernatants were evaporated to drynes and resuspended in a small volume of petrolether. This crude extract was spotted on thin layer plates (Silica GF_{254}, 0.5 mm) and developed with petrolether/diethylether (99.5/0.5) (solvent system 1). Bound sulfide with an R_F of 0.84 was scraped off the plates, eluted with hot methanol, and spotted on a second plate which was developed with the solvent system 2 (acetone/water, 1/9) where bound sulfide stayed at the origin. The bound sulfide was eluted from the gel and spotted again on a TLC plate which was developed with ethylacetate (solvent system 3; R_F = 0.86). The last plate was prerun with acetone and ethylacetate to remove impurities. This material was used for the identification procedures.

Detection of Bound Sulfide. Bound sulfide can be detected on silica gel GF_{254} plates by quenching of the fluorescence as a blue spot on the green background using a 254 nm light source. Detection was possible also using a Turner fluorimeter 111 equipped with a Camag-T-scanner using filter 110-810 for the primary light source and filter 110-823 (10%) for the photomultiplier side. The obtained signal was recorded in a W & W recorder 1100. Spraying with Rhodamin 6B (0.005% in methanol) yields a red spot on a pink background (26). After spraying with sodium azide in iodine the bound sulfide can be detected as a white spot on a yellow-brown background (4).

For the experiments using labeled sulfate, the isolation procedure was terminated after the separation on the first solvent system. Radioactivity was detected using a TLC scanner LB 2720/2031 from Berthold (München, West Germany). Quantitative measurements were made by elution of bound sulfide and counting in a liquid scintillation counter Beckman LS 100 using a Rotiscint cocktail.

Determination of Bound Sulfide by Reduction to Free Sulfide. Reduction of bound sulfide to free sulfide was achieved with dithioerythritol (DTE) using the following conditions (μmol): Tris free base, 100; DTE, 5; and extract as needed in a total

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volume of 1 ml. Incubation for 20 min at 37°C. Hydrogen sulfide formed was determined according to (33); however, an ϵ of 19.3 $\times 10^6$ cm^2 $\times$ mol^{-1} was used for normalized conditions with 400 μl methanol in the test.

Formation of Thiocyanate. Bound sulfide reacted with cyanide forming thiocyanate. The following test conditions were used (μmol): Tris-HCl (pH 9.0), 100; KCN, 20; and bound sulfide as needed in a total volume of 1 ml. The test was stopped after 15 min incubation at 80°C by adding acidic $FeCl_3$ (34); using a ϵ of 1.96×10^6 cm^2 $\times$ mol^{-1} at 460 nm for the red FeSCN complex.

UV-Visible Spectroscopy. A Beckman spectrophotometer DU 7 was used connected with a Hewlett-Packard 2672 plotter.

High Pressure Liquid Chromatography (HPLC). Bound sulfide was assayed by reverse-phase HPLC on a stainless steel column (250 × 4.0 mm; Knauer, Berlin, West Germany) filled with μBondapak-C_8 (Merck, Darmstadt, West Germany) and the solvent system methanol/water (85/15). A Rheodyne 7120 injection system (Latek, Heidelberg, West Germany), a 600 A pump from Waters (Königstein, West Germany) was used at a flow rate of 1.5 ml/min. Bound sulfide was detected with a 8-μl flow through cell with 1 cm path at 263 nm. For preparative analysis, the detector was connected with a fraction collector. Elemental sulfur was analyzed as sulfide after reduction with DTE as described above.

Mass Spectrometry (MS). Mass spectra of the purified sample and authentic sulfur flower were recorded on a Varian CH 7A mass spectrometer (70 ev, datasystem SS-188 MS).

Formation of Cysteine. Formation of cysteine was analyzed by incubation of cysteine synthase and *O*-acetyl-L-serine with bound sulfide isolated from *Chlorella* (S°) in the presence and absence of thiols. Cysteine was determined as the red ninhydrin complex (9) modified as described (20). Cysteine synthases from *Chlorella* and spinach were purified as described earlier (28, 29).

Chemicals. [^{35}S]Sulfate was purchased from Buchler (Braunschweig, West Germany). *O*-Acetyl-L-serine, L-cysteine ethylester, and L-cysteine methylester were obtained from Sigma (München, West Germany) and elemental sulfur from Unfried (München, West Germany). Rotiszint and DTE were ordered from Roth (Karlsruhe, West Germany) and *p*-amino-dimethylanilin from Serva (Heidelberg, West Germany). All other chemicals not mentioned were purchased from Merck.

RESULTS

Isolation Procedure of Bound Sulfide. *Chlorella* cells grown on L-cysteine ethylester contained high amounts of bound sulfide, which could be liberated as free sulfide after reduction by different thiols. The following procedure has been used for the isolation of bound sulfide. Cells were extracted with hot methanol; further purification was achieved on silica gel using solvent system one, because this system separated the Chl from bound sulfide. The second solvent system was used to separate lipids from bound sulfide (26) which were not separated in solvent system 1. The solvent system 3 was used as the final preparative step prior to chemical analysis.

Chemical Properties of Bound Sulfide and Its Identification as Elemental Sulfur (S°). Bound sulfide was run in different TLC systems on silica gel GF_{254}; the different solvent systems and the corresponding R_F values are summarized in Table I. The R_F values obtained suggested that this substance could be elemental sulfur; therefore, a cochromatography of bound sulfide with authentic sulfur flower was performed, and one example is shown in Figure 1, in which elemental sulfur and bound sulfide cochromatographed in solvent system 1. Identical R_F values and cochromatography of bound sulfide and elemental sulfur were found in the other solvent systems of Table I also. Separation behavior of bound sulfide and elemental sulfur was also identical using reversed phase HPLC (Figure 2) with the solvent system methanol/water (85/15).

Table I. *R_F Values for Bound Sulfide*

Analytical plates with silica gel GF_{254} (0.25 mm) were used. Bound sulfide was detected by UV quench or formation of hydrogen sulfide.

Solvent System		R_F
1. Petrolether (b.p. 40–65°C)/diethylether	99.5/0.5	0.84
2. Acetone/water	1/9	0.00
3. Ethylacetate		0.86
4. Petrolether/diethylether/acetic acid	9/1/0.1	0.68
5. Petrolether		0.57
6. Acetone/water	9/1	1.00
7. Acetone/water	1/1	0.00
8. *n*-Hexane		0.88
9. *n*-Heptane		0.84
10. cyclo-Hexane		0.81
11. Chloroform/methanol	9/1	0.88
12. Chloroform/methanol/water	46/18/3	0.85
13. Chloroform/methanol/acetic acid/water	85/25/15/3	1.00
14. Chloroform/*n*-hexane	2/3	0.96

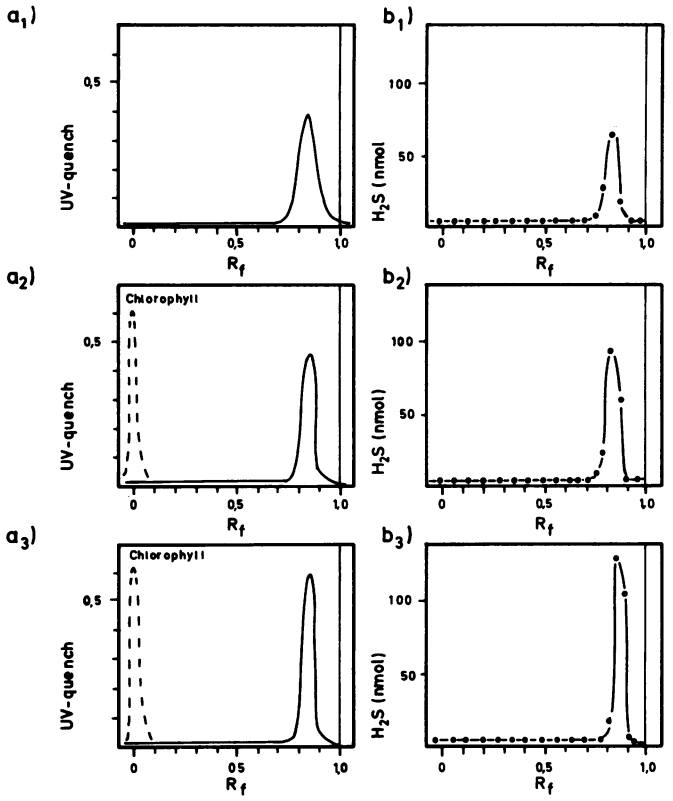

FIG. 1. Thin-layer chromatography of bound sulfide extracted by hot methanol. Silica-gel GF_{254} (0.25 mm); solvent petrolether/diethylether 99.5/0.5. (a), Detection by UV quench; (b), detection by formation of hydrogen sulfide; (1), methanol extract of *Chlorella* grown on sulfate after addition of sulfur-flower; (2), methanol extract of *Chlorella* grown on L-cysteine ethylester; (3), cochromatography of 1 and 2.

The solubility of this bound sulfide was analyzed using different organic solvent systems. Poor solubility was found in chloroform or benzene; moderate solubility was detected in hot methanol, ethanol, or *n*-hexane; whereas good solubility was detected using ethylacetate, petrolether, and especially carbon disulfide.

Further evidence for elemental sulfur was obtained from the reactions with thiols forming free sulfide and the formation of thiocyanate after incubation at 80°C with cyanide; a ratio of

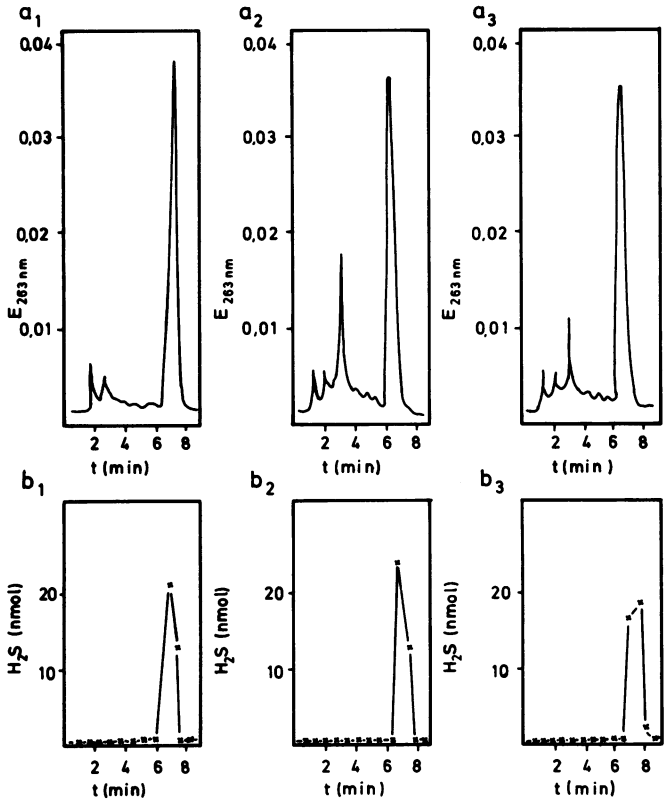

FIG. 2. High pressure liquid chromatography of bound sulfide. Stainless steel column (4 × 250 mm) packed with μBondapak-C_8 was used; methanol/water (85/15) was the eluent with a flow rate of 1.5 ml/min at 22°C. The symbols used and the material are identical to Figure 1.

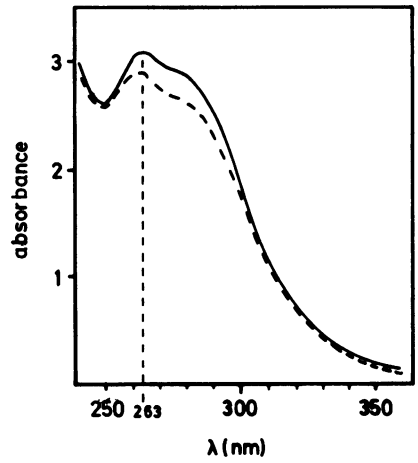

FIG. 3. UV-visible spectra of bound sulfide and sulfur (S°). (——), Methanol extract containing bound sulfide; (- - -), sulfur-flower dissolved in methanol.

sulfide/SCN of 1/1.05 was obtained (data not shown). The absorption spectrum of bound sulfide in comparison with authentic elemental sulfur is shown in Figure 3; both fractions showed a relative absorption maximum at 263 nm, which explains that this compound can be detected on silica gels GF_{254} using UV light. The extinction coefficient in methanol was determined to $\epsilon_{263} = 3.29 \times 10^6$ cm^2 × mol^{-1}. The identity of bound sulfide with elemental sulfur was demonstrated using MS. For comparison of bound sulfide with elemental sulfur, sulfur powder was added to sulfate-grown *Chlorella* cells, the purification procedure was performed as described in "Materials and Methods," and a blank (Fig. 4B) was obtained using the same procedure without addition of sulfur powder (Fig. 4C). These data are summarized in Figure 4. Good agreement was found for the mol wt of 256 m/e according to S_8 rings of elemental sulfur (Fig. 4D). The other fractions of 224 m/e to 64 m/e are fragments of S_7 to S_2 in relation of S_8:S_7:S_6:S_5:S_4:S_3:S_2 to 84%:3.2%:36%:53%:64%:22%:100%. Additional fragments with the mass M + 2 are seen such as 258 m/e, because S° is an A + 2 element. The background of Figure 4C isolated from sulfate-grown cells without the addition of elemental sulfur shows a significant mass of 256 m/e, which might be elemental sulfur as well, since this spectrum was optimized for maximal appearance of the mass of 256. No elemental sulfur, however, was detected in crude extracts obtained from sulfate-grown *Chlorella* cells, which might be a problem of the detection limits of the methods used. The data presented here demonstrate that bound sulfide is identical with elemental sulfur and that it is not a polysulfide linked to a thiol group or a polythionate.

Metabolism of Elemental Sulfur. *Chlorella* cells accumulated elemental sulfur in the early logarythmic growth phase when L-cysteine ethylester was used as a sulfur source whether sulfate was present or absent (Fig. 5). Maximum values of 18 μmol (S)/mg Chl were obtained. However, when cells reached the stationary phase, elemental sulfur clearly disappeared, demonstrating that *Chlorella* cells have the capacity for formation and reutilization of elemental sulfur. It was possible to demonstrate *in vitro* the formation of cysteine from elemental sulfur (isolated from *Chlorella*) and *O*-acetyl-L-serine in the presence of a thiol and cysteine synthase purified from spinach (Table II) or a crude or purified preparation from *Chlorella* (data not shown). Cysteine formation under these conditions was found only in the presence of thiols, suggesting a reduction of this elemental sulfur preparation to free sulfide prior to cysteine biosynthesis. This was substantiated by the finding that this elemental sulfur is split by different thiols to free sulfide (Table III); thus, natural thiols such as glutathione or cysteine will split elemental sulfur to hydrogen sulfide. Ratios for H_2S/SCN/cysteine of 1.05/1/1.05 were found when these three reactions were run side by side, which clearly demonstrates that a thiol-dependent reduction of elemental sulfur and coupling to cysteine biosynthesis can be demonstrated *in vitro* suggesting its occurrence also *in vivo*. Elemental sulfur isolated from *Chlorella* cells grown on radiolabeled sulfate and L-cysteine ethylester did not contain radioactivity, suggesting that the elemental sulfur formed was derived only from L-cysteine ethylester. This is in accord with our observation for an accumulation of elemental sulfur during growth of *Chlorella* on L-cysteine ethylester as the only sulfur source.

DISCUSSION

The green alga *C. fusca* has the capacity to metabolize a variety of sulfur compounds for growth (Krauss *et al.*, submitted). Some sulfur-containing substances—especially L-cysteine ethylester and L-cysteine methylester—led to the accumulation of bound sulfide, which was characterized as elemental sulfur by chemical and MS measurements.

This bound sulfide was extracted from *Chlorella* cells in hot methanol. After evaporation of the methanol, this bound sulfide was taken up as a hydrophobic sulfide in different solvent systems with the best solubility in carbon disulfide, which is the best solvent for elemental sulfur (34% at ambient temperature). A second form of bound sulfide, a polysulfide fraction, was obtained, which was not soluble in CS_2, which, however, was soluble either in water or methanol. This polysulfide fraction was discharged. The bound sulfide consists of CS_2-soluble sulfur as described for S_λ or S_α (22, 36, 39), whereas the polysulfide fraction was not soluble in CS_2. Therefore, the polysulfide fraction could

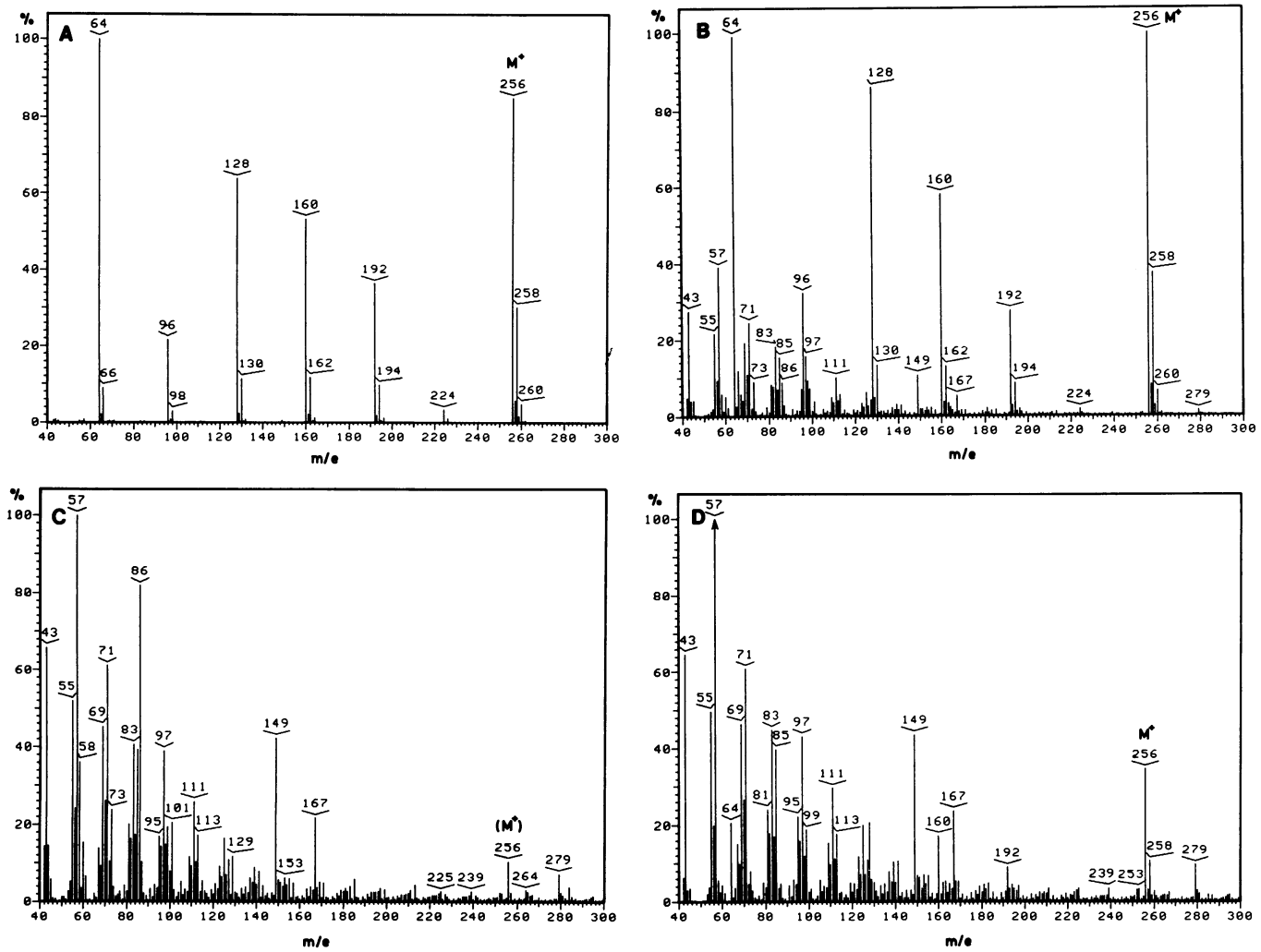

FIG. 4. Mass spectrometry of bound sulfide and sulfur-flower used after separation with solvent three. (A), Sulfur-flower; (B), sulfur-flower added to methanol extract of *Chlorella* grown on sulfate; (C), methanol extract of *Chlorella* grown on sulfate; (D), methanol extract of *Chlorella* grown on L-cysteine ethylester and sulfate.

be a polysulfide, a polythionate, or S_μ, an allotrophic from of S° stable only at ambient temperature under certain conditions (35, 39). Such amorphous S° is believed to consist of many linear sulfur atoms as a polythionate with the general formula $H_2S_xO_6$. The isolated polysulfide fraction has not been analyzed in detail so far, since less of this material was available. Separation of the polysulfide fraction from bound sulfide was also achieved in solvent system 1, which was used to separate Chl and carotinoids from elemental sulfur.

Elemental sulfur and bound sulfide migrated identical to the systems described by Davies and Thuraisingham (4). The chemical nature for this compound being elemental sulfur was confirmed by spraying the chromatograms with Rhodamine 6B, normally used for lipid detection (26). Elemental sulfur and bound sulfide appeared as a red spot. A white spot was obtained on a yellow-brown background when sodium azide and iodine were used for detection (4). Normally, S° is identified only by one of several methods necessary for identification of elemental sulfur; interferences especially by polythionates and other thiol compounds are evident. Therefore, all test systems were combined using biochemical as well as spectroscopic methods including MS for the identification of this bound sulfide as elemental sulfur.

The absorption spectrum of elemental sulfur and bound sulfide in methanol (shown in Fig. 3) is typical for elemental sulfur with an absorption maximum at 263 nm; this maximum is not found for polythionates (2). This maximum was found using methanol, ethanol, acetone, chloroform, and *n*-hexane (5). The system normally used for elemental sulfur determination is its reaction with cyanide forming thiocyanate at high temperature. But this test is not specific for S° because thiocystine (7), polythionates, polysulfides, disulfides, and tetrasulfides react in the same manner with cyanide forming thiocyanate (8). Nucleophilic attack of S° by thiols was also used with incubation times of 15 to 20 min at 37°C (16). The data of this paper (Table II) clearly demonstrate that elemental sulfur can be split by thiols to hydrogen sulfide; however, high levels of thiosulfate (33) and cysteine as well as glutathione and mercaptoethanol inhibit the determination of sulfide by the methylene blue method, whereas according to our own data little interference was found using dithioerythritol at 2 mM concentrations.

Convincing data showing the identity of bound sulfide with elemental sulfur were obtained using MS (Figure 4). The fragment pattern and molecular ion of 256 m/e is typical for S_8 (22). Cyclic polysulfides observed in higher plants (11) are excluded because their fragments are quite different from that of bound sulfide. This does, however, not indicate that the S_8 configuration is formed *in vivo*, since other allotrophic forms are converted

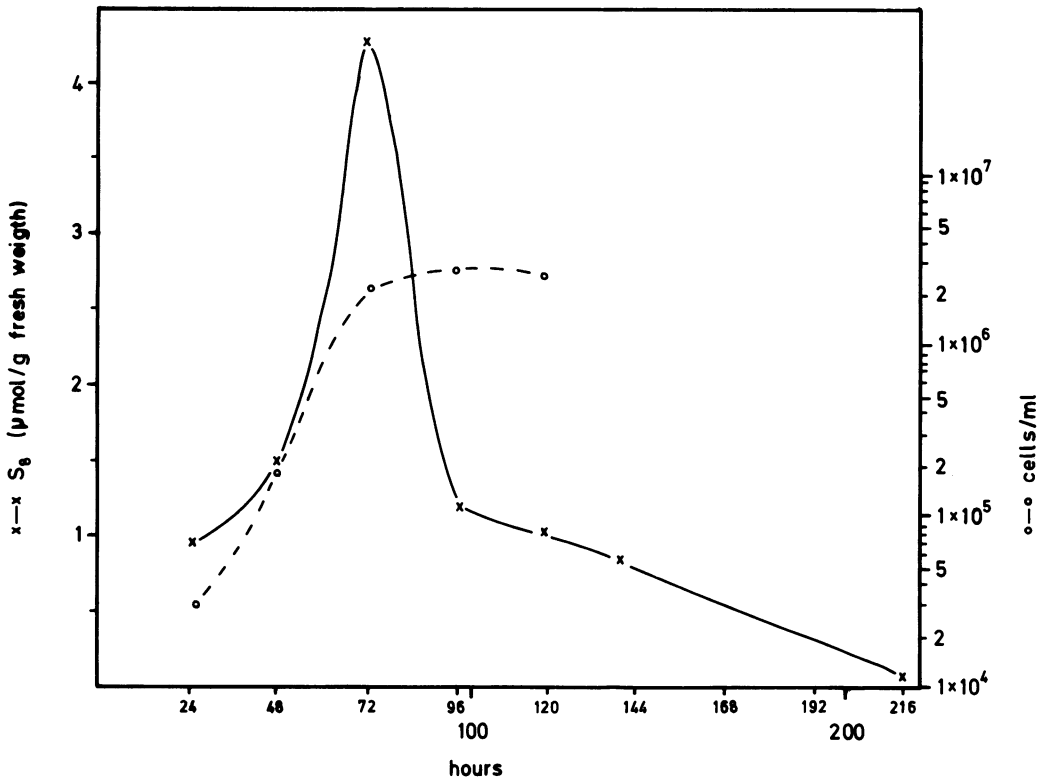

FIG. 5. Formation of bound sulfide by *Chlorella* cells growing on L-cysteine ethylester and sulfate. Samples were taken as indicated, centrifuged, and resuspended in 3 volumes of 20 mM Tris-HCl (pH 8.0)/0.1 m $MgCl_2$. Bound sulfide was determined with the following test conditions (µmol): Tris free base, 100; DTE, 2; whole cells as needed in a total volume of 1 ml. Incubation for 20 min at 37°C. Hydrogen sulfide formed was determined with the methylene blue method.

Table II. *Formation of Cysteine by Reaction of Elemental Sulfur with Dithioerythritol (DTE) and Cysteine Synthase*

The following test conditions were used (µmol): Tris-HCl (pH 7.5), 100; DTE, 2, Na_2S, 5; *O*-acetyl-L-serine (OAS) (5); bound sulfide (isolated from *Chlorella*), 189 nmol; cysteine synthase (CS) purified from spinach, 640 µg in a total volume of 1 ml. Incubation for 30 min at 37°C. Cysteine formed was determined as the red ninhydrin complex at 560 nm.

Test	Additions					Cysteine Formed
	DTE	Na_2S	Bound Sulfide	OAS	CS	
						nmol
a	+	+	−	+	+	360
b	+	+	+	+	−	10
c	+	+	+	−	−	20
d	−	−	+	+	+	41
e	+	−	+	+	+	158
f	+	+	+	+	+	360

Table III. *Reaction of Elemental Sulfur with Some Thiols*

Test conditions (µmol): Tris-HCl (pH 9), 100; thiol as indicated, 2 mM; dissolved in 20 mM Tris-HCl (pH 9.0); S°, 109 nmol dissolved in 100 µl methanol in a final volume of 1 ml. Incubation for 20 min at 37°C. The formation of hydrogen sulfide was determined as methylene blue at λ = 670 nm (final volume, 1.2 ml). For calibration values, 100 nmol of hydrogen sulfide dissolved in 1 mM EDTA was tested instead of S° in the presence of the thiol to calibrate the system.

Thiol Compound Added	Methylene Blue Formation from H_2S in the Presence of Thiols		Methylene Blue Formation from S° in the Presence of Thiols		Hydrogen Sulfide formed from S°	
	ϵ_{670}	%	ϵ_{670}	%	*nmol*	%
Dithioerythritol (DTE)	1.320	100	1.289	100	97.6	100
1,2-Dimercaptopropanol (BAL)	1.243	94	1.243	94	97.5	99
2-Mercaptoethanol (ME)	0.550	42	0.462	36	84.0	86
Glutathione reduced (GSH)	0.473	36	0.383	30	80.9	83
L-Cysteine	0.345	26	0.361	28	104.6	107
Without thiol	0.743	56				

rapidly to S_8 (22). Sulfur globules detected in procaryotes are described containing orthorhombic sulfur (36) or fluid sulfur (12). Normally, elemental sulfur found in organisms is not surrounded by a lipid layer but is confined to a membrane system of proteins (24).

Chlorella cells accumulated S° during growth on the L-cysteine esters, however, not during growth on L-cysteine. The reason for the discrimination between L-cysteine and L-cysteine ethyl- and methylesters is not understood, although one might speculate that the esters are not seen as free cysteine. Therefore, the esters might be transported into the cell without a regulatory feedback signal. A second possibility would be related to the change of the pI of these cysteine derivatives. That the pI of a substance could influence the uptake system was shown for the pairs glutamate/glutamine and aspartic acid/asparagine (3). Another possibility would be that these cysteine esters are transported into the cell by a different transport system not related to a cysteine transport system; in this case, the regulatory mechanism for a controlled cysteine accumulation would regulate the wrong uptake system.

The possible routes for the metabolism of L-cysteine ethylester once accumulated within the cell are summarized in Figure 6. We have indicated evidence for each reaction by a reference to the organism where such a reaction was detected.

The identification of elemental sulfur in the green alga *Chlorella* raises questions concerning the distribution of elemental sulfur in photosynthetic organisms. Elemental sulfur is formed during growth of phototrophic bacteria on sulfide (12, 13, 36). It has been detected in cyanobacteria as an oxidation product during anoxigenic photosynthesis with only PSI active. So far, only two reports for the formation of elemental sulfur from

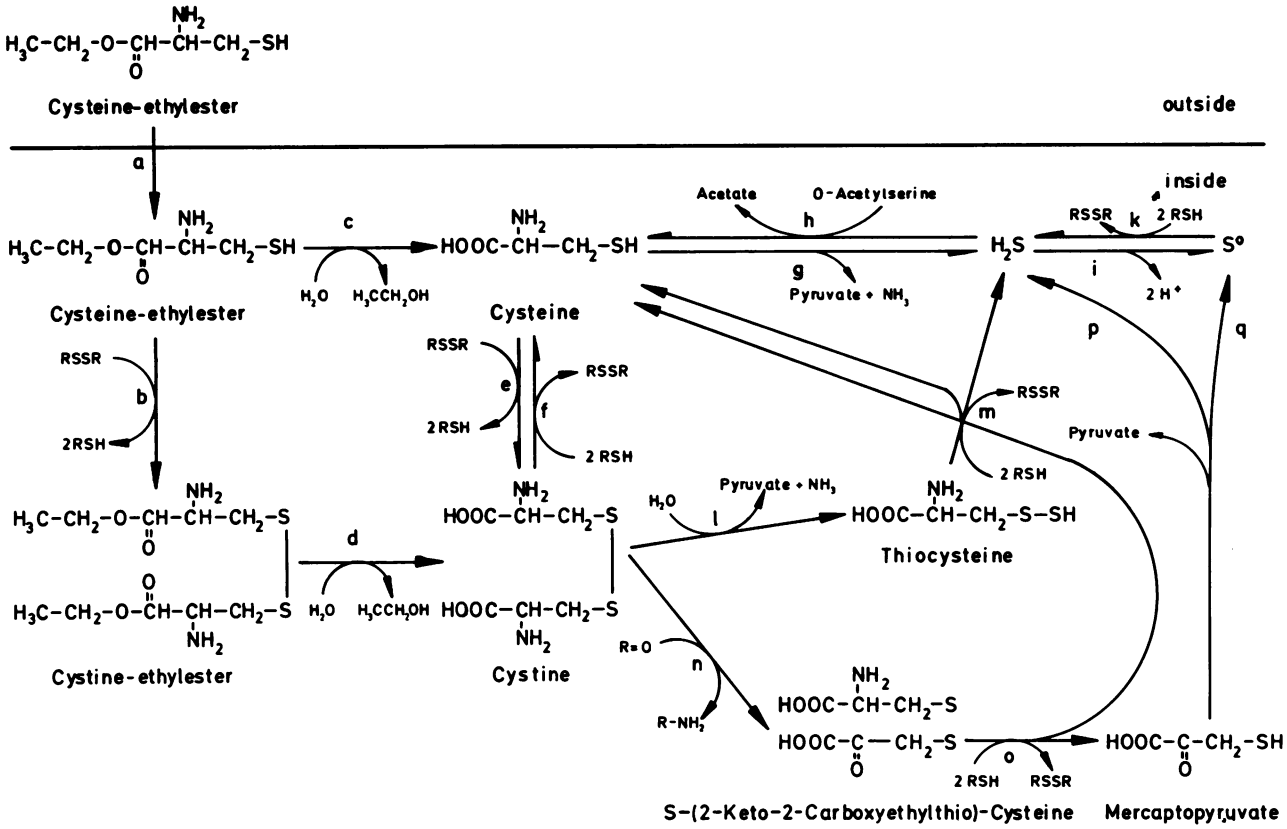

FIG. 6. Suggested routes for metabolism of L-cysteine ethylester in *Chlorella*. Evidence for these reactions is summarized as follows: (a), Cysteine uptake, tobacco cells (Harrington and Smith, 14); (b) cysteine oxidase, *Synechococcus 6301* (Schmidt and Krämer [31], which is present in *Chlorella* [unpublished]); (c) carboxylesterase, different plants (Schwartz *et al.*, 32); (d) carboxylesterase, see (c); (e), cysteine oxidase, see (b); (f), cysteine reductase, pea[2] (Romano and Nickerson, 27; Hatch and Turner, 15); (g), cysteine desulfhydrase, *Chlorella* (Schmidt and Erdle, 30); (h), cysteine synthase, *Chlorella* (Schmidt, 27); (i), (1) anoxygenic photosynthesis, cyanobacteria (Garlik *et al.*, 10); or (2) oxidation by cytochromes, *Chromatiaceae* and *Chlorobiaceae* (Fischer, 6); (k) reduction, *Chlorella* (see Table III of this paper); (l), cystine lyase, turnip roots (Mazelis *et al.*, 21); (m) evidence from turnip roots (Anderson and Thompson, 1; rhodanese?); (n), cysteine transamination, rat (indirect evidences, Ubuka *et al.*, 37); (o), chemical reduction by thiols, see (f); (p), mercaptopyruvate reductase, *Escherichia coli* (Kondo *et al.*, 19); (q) mercaptopyruvate sulfotransferase, *E. coli* (Vachek and Wood, 38; rhodanese?).

eucaryotic photosynthetic organisms have appeared for the red algae *Erythrophyllum and Ceramium* (17, 18).

It is not clear from our studies if elemental sulfur is only formed using artificial thiols for growth. It is clear, however, that the capacity for a storage of reduced sulfur as elemental sulfur has not been lost during the evolution within a green alga.

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[2] Unpublished results from our laboratory indicate that cystine reduction to cysteine can be catalyzed by thioredoxin and thioredoxin reductase in plants.

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